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Total Synthesis of 17,17,18,18-d_4-$iPF_{2\alpha}$-VI and Quantification of $iPF_{2\alpha}$-VI in Human Urine by Gas Chromatography/Mass Spectrometry

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Isoprostanes are a new class of natural products formed in humans as a result of free-radical-catalyzed lipid peroxidation of polyunsaturated fatty acids. These endogenous compounds are isomeric with biologically active prostaglandins and have great promise as markers of oxidant stress *in vivo*. $iPF_{2\alpha}$-III (previously 8-*iso*-$PGF_{2\alpha}$), an isoprostane from Class III (previously known as Class IV), has been used as an index of free-radical-induced oxidative stress. This isoprostane is also produced by the cyclooxygenase enzymes COX1 and COX2. We are proposing a new reliable index of oxidative stress based on $iPF_{2\alpha}$-VI (previously $IPF_{2\alpha}$-I), a new Class VI isoprostane we recently discovered. The advantages of $iPF_{2\alpha}$-VI are that it is several fold more abundant in urine than $iPF_{2\alpha}$-III, hence allowing more accurate determinations. Equally, the proximity of the C-5 OH function to the carboxylic acid allows the formation of the lactone 35 which is easier to purify from other iPs which cannot form such lactones. We have performed the first total synthesis of d_4-$iPF_{2\alpha}$-VI by using two synthons, (3,3,4,4-d_4)-hexylphosphonium bromide 23 prepared from 5-hexynol and *syn-anti-syn* lactone 25 synthesized from D-glucose. We have developed two variants of a sensitive GC/MS assay using the synthetic d_4-$iPF_{2\alpha}$-VI as an internal standard to determine the levels of endogenous $iPF_{2\alpha}$-VI in biological fluids. Quantification of $iPF_{2\alpha}$-VI formed *in vivo* may be a more reliable index to assess oxidant stress in humans.

Isoprostanes (iPs)[2] are a new class of natural products formed in humans by free-radical-catalyzed lipid peroxidation of arachidonic acid (AA) (1). AA is usually esterified to phospholipids and then released into circulation by the action of phospholipases (2). We and others have predicted that four classes of isoprostanes would be formed by the free-radical-induced peroxidation of AA (Fig. 1) (3–6). Six classes of iPs have been predicted to be formed from eicosapentaenoic acid (EPA) (5, 7). The $iPF_{2\alpha}$-III (8-*iso*-$PGF_{2\alpha}$), a Type III isoprostane (previously Type IV) as shown in structure **5A**, Fig. 1, is a vasoconstrictor (8) and a minor product of the action of cyclooxygenases (COX) 1 and 2 on AA (9, 10, 34).

The measurement of iPs as an index of oxidant stress has been performed in a number of situations. Elevated levels of $iPF_{2\alpha}$-III have been observed in liver diseases (11), cigarette smoking (12, 13, 35), poisoning with paraquat and paracetemol (14), coronary reperfusion (15), diabetes (16), and low-density lipoprotein (LDL) oxidation (17–20). Most recently, using the methods described in this article, we have measured elevated levels of $iPF_{2\alpha}$-VI **34**, a class VI isoprostane (Fig. 1), in cigarette smokers, copper-catalyzed LDL oxidation (20), acute coronary angioplasty (15), and the antiphospholipid antibodies of (aPL)-positive patients (21). We have also shown the presence of $iPF_{2\alpha}$-VI in human atherosclerotic plaques and the elevation of

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[2] Abbreviations used: iPs, isoprostanes; AA, arachidonic acid; $iPF_{2\alpha}$-III, a Type III isoprostane; COX, cyclooxygenase; LDL, low-density lipoprotein; $iPF_{2\alpha}$-VI, a Type VI isoprostane; TMS, tetramethylsilane; THF, tetrahydrofuran; DIPEA, *N,N*-diisopropylethylamine; PFB, pentafluorobenzyl; DCC, *N,N'*-dicyclohexyl-carbodiimide; EDCM, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide; BSTFA, bis-(trimethylsilyl)trifluoroacetamide; SPE, solid-phase extraction; LTC_4, leukotriene C_4.

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this isoprostane in atherosclerotic plaque obtained at endarterectomy (22).

We have previously reported the first total synthesis of iPF$_{2\alpha}$-VI **34** and the identification of this isoprostane in human urine (23). With the discovery of this novel isoprostane, which is also the first positive proof for the existence of Group VI isoprostanes in humans, we decided to synthesize the deuterium-labeled iPF$_{2\alpha}$-VI in order to use it as an internal standard to quantitate iPF$_{2\alpha}$-VI levels in various biological fluids by GC/MS or LC/MS. Here we report the first total synthesis of 17,17,18,18-tetradeuterated-iPF$_{2\alpha}$-VI **33** and its use in the development of an assay for the quantification of iPF$_{2\alpha}$-VI **34** in human urine by GC/MS.

MATERIALS AND METHODS

1. Synthesis

NMR spectra were recorded on a Bruker AMX-360 NMR spectrometer. Proton chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) unless noted otherwise. All reactions were monitored by thin-layer chromatography (TLC) carried out on 0.25-mm E. Merck silica gel plates (60 F_{254}) with a fluorescent indicator, using UV light as visualizing agent and/or ethanolic *p*-anisaldehyde solution (5%), ethanolic phosphomolybdic acid (10%) solution, and heat as developing agents. Flash chromatography was performed using E. Merck silica gel 60 (200–400 mesh). All nonaqueous reactions were carried out under an argon atmosphere with dry, freshly distilled solvents under anhydrous conditions unless otherwise noted. Tetrahydrofuran (THF) was distilled from sodium/benzophenone. Dichloromethane, diethyl ether, benzene, and toluene were distilled from calcium hydride and pyridine from barium oxide. Yields refer to chromatographically and spectroscopically (^{1}H NMR) homogeneous materials, unless otherwise noted. Most reagents were purchased from commercial suppliers and used without further purification unless otherwise noted.

*Tetrahydro-2-(3-hexynyloxy)-2H-pyran (**19**)*

A mixture of 3-hexynol **17** (2 g, 20 mmol) and *p*-toluenesulfonic acid (38 mg, 0.2 mmol) was stirred and 3,4-dihydro-2H-pyran **18** (2.018 g, 24 mmol) added over a period of 5 min while the temperature was maintained at 25°C and the reaction mixture stirred at this temperature for an extra 30 min. The product was purified by flash chromatography eluting with 10% ethyl acetate in hexane to afford hexynyl ether **19** (3.655 g, 99%) as a colorless liquid: R_f = 0.63 (15% ethyl acetate in hexane).

*Tetrahydro-2-(3,3,4,4-d$_4$-hexanyloxy)-2H-pyran (**20**)*

Hexynyl ether **19** (1 g, 5.49 mmol) in anhydrous benzene (25 ml) was placed in a flask connected to a deuteration apparatus, the air was removed, and the system was flushed with argon twice. The catalyst, tris(triphenylphosphine)chlororhodium (0.25 g, 0.27 mmol), was added and the system was degassed and flushed with deuterium (99.98%) twice. The reaction mixture was stirred at 25°C under deuterium atmosphere until no more deuterium was taken up (8 h). The catalyst was filtered off through a Celite mat and the filtrate was concentrated *in vacuo*. The residue was passed through a column of silica gel, eluting with 30% diethyl ether in hexane to yield hexyl-3,3,4,4-d$_4$ ether **20** (1.01 g, 97%) as a colorless liquid: R_f = 0.58 (15% ethyl acetate in hexane).

*3,3,4,4-d$_4$-Hexan-1-ol (**21**)*

To a solution of hexyl-3,3,4,4-d$_4$ ether **20** (0.540 g, 2.84 mmol) in anhydrous methanol (10 ml) at 25°C was added *p*-toluenesulfonic acid (25 mg, 0.13 mmol). The reaction mixture was stirred at 25°C for 1.5 h. The solvent was removed *in vacuo* (not to dryness). The residue was poured into crushed ice and the resulting mixture was extracted with diethyl ether (2 × 10 ml). The combined extracts were dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography, eluting with 25% diethyl ether in hexane to afford d$_4$-hexanol **21** (0.24 g, 80%) as a colorless liquid: R_f = 0.135 (15% ethyl acetate in hexane).

*3,3,4,4-d$_4$-1-Bromohexane (**22**)*

To a solution of d$_4$-hexanol **21** (0.24 g, 2.26 mmol) in anhydrous methylene chloride (10 ml) at 0°C was added dibromophosphorane (1.05 g, 2.49 mmol) in three portions. The reaction mixture was allowed to warm to room temperature and stirred at room temperature for 1 h. The reaction solution was washed with 5% aqueous $NaHCO_3$ (5 ml) and with brine (5 ml). The organic layer was separated, dried over Na_2SO_4, and concentrated to 2 ml *in vacuo*. The residue was diluted with hexane (10 ml) and the solid precipitate was filtered off through a Celite mat. The filtrate was dried with Na_2SO_4. The removal of the solvent afforded d$_4$-bromohexane **22** (0.31 g, 81%) as a colorless liquid.

*3,3,4,4-d$_4$-Hexyltriphenylphosphonium Bromide (**23**)*

To a solution of d$_4$-hexyl bromide **22** (0.31 g, 1.82 mmol) in anhydrous acetonitrile (10 ml) at 25°C was added triphenylphosphine (1.1 g, 3.64 mmol). The reaction mixture was refluxed for 22 h. The solvent was removed *in vacuo* and the residue was purified by flash

chromatography using 0.5% methanol in methylene chloride to yield 3,3,4,4-d_4-hexyltriphenylphosphonium bromide (**23**) (0.68 g, 85%) as a white solid: R_f = 0.34 (10% methanol in methylene chloride).

^1H NMR ($CDCl_3$). δ7.9–7.77 (m, 9H, 3 × (C_3-H, C_4-H, and C_5-H), aromatic), 7.74–7.67 (m, 6H, 3 × (C_2-H and C_6-H), aromatic), 3.8 (dt, J = 8.2 and 12.9 Hz, 2H, C_1-H_2), 1.6 (bq, J = 8.9 and 16.4 Hz, 2H, C_2-H_2), 1.23 (bq, J = 7.5 and 14.9 Hz, C_5-H_2), 0.82 (t, J = 7.4 Hz, 3H, C_6-H_3).

^{13}C NMR ($CDCl_3$). δ135.01 (3 × C_1, aromatic), 134.98 (3 × C_4, aromatic), 133.77 (3 × C_2, aromatic), 133.66 (3 × C_6, aromatic), 130.51 (3 × C_3, aromatic), 130.37 (3 × C_5, aromatic), 118.62 and 117.67 (C_1), 22.98 (C_2), 21.82 (C_5), 13.75 (C_6).

*1β-[3α,5α-Bis(tert-butyldimethylsilyl)oxy-2β-(2(Z)-5,5,6,6-d_4-octenyl)-cyclopentyl]-methanol (**27**)*

To a suspension of d_4-hexyltriphenylphosphonium bromide **23** (90.25 mg, 0.186 mmol) in anhydrous THF (1 ml) at 25°C was added 1 M THF solution of potassium *tert*-butoxide (168 ml, 0.168 mmol) dropwise. The resulting red solution was stirred at 25°C for 15 min and then cooled to −78°C. To the resulting yield was added HMPA (0.2 ml) and the mixture was stirred at −78°C for 5 min. To this was added the lactol **26** (15 mg, 0.0373 mmol) in anhydrous THF (0.3 ml). The resulting solution turned orange in color. The reaction mixture was stirred at −78°C for 1 h and then allowed to warm to 0°C in 1 h and further stirred at room temperature for 10 min. The reaction mixture was quenched with 5 wt% aqueous $KHSO_4$ (1 ml) and diluted with EtOAc (5 ml). The organic layer was separated, washed with brine (5 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash column chromatography eluting with ethyl acetate/hexane (5/95) to afford the d_4-olefinic compound **27** (16 mg, 90%) as an oil: R_f = 0.72 (20% EtOAc in hexane).

*1β-[3α,5α-Bis(tert-butyldimethylsilyl)oxy-2β-(2(Z)-5,5,6,6-d_4-octenyl)-cyclopentyl]-carbaldehyde (**28**)*

To a solution of oxalyl chloride (48.5 μl, 0.0968 mmol, 2 M/CH_2Cl_2) in anhydrous methylene chloride (1 ml) at −78°C was added anhydrous dimethyl sulfoxide (11.34 mg, 0.145 mmol). The resulting mixture was stirred at −78°C for 10 min and to this was added a solution of the d_4-alcohol **27** (23 mg, 0.0484 mmol) in anhydrous methylene chloride (0.3 ml). The resulting mixture was stirred at −78°C for 20 min and then anhydrous triethylamine (29.4 mg, 0.29 mmol) was added. The reaction mixture was stirred at −78°C for 20 min and then allowed to warm to 0°C in 20 min. The reaction was quenched with a cold saturated aqueous NH_4Cl (5 ml) solution. The organic layer was separated and the aqueous layer was extracted with methylene chloride (2 × 5 ml). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash chromatography using ethyl acetate/hexane (5/95) as an eluent to yield the d_4-aldehyde **28** (20 mg, 88%) as a colorless oil: R_f = 0.77 (5% EtOAc in hexane).

*Methyl (6E,8β,9α,11α,14Z)-9,11-Di(tert-butyldimethylsilyl)oxy-17,17,18,18-d_4-5-oxo-prosta-6,14-dienoate (**30**)*

To a solution of the β-keto phosphonate **29** (21.3 mg, 0.0845 mmol) in anhydrous THF (1 ml) at 25°C was added sodium hexamethyldisilazane (0.845 ml, 0.0845 mmol, 1 M/THF) and the solution was stirred at 25°C for 15 min and then cooled to −78°C. To the resulting anion of the β-keto phosphonate at −78°C was added the d_4-aldehyde **28** (20 mg, 0.136 mmol) in anhydrous THF (0.3 ml). The reaction mixture was stirred at −78°C for 10 min and then allowed to warm to 0°C in 1 h and further stirred at 0°C for 7 h. The reaction was quenched with saturated aqueous NH_4Cl (5 ml). The resulting mixture was extracted with ethyl acetate (2 × 7 ml). The combined organic extracts were washed with brine (5 ml), dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash chromatography eluting with ethyl acetate/hexane (5/95) to afford the d_4-α,β-unsaturated ketone **30** (24 mg, 95%) as a colorless oil: R_f = 0.37 (15% EtOAc in hexane).

*Methyl (5S,6E,8β,9α,11α,14Z)-9,11-Di(tert-butyldimethylsilyl)oxy-17,17,18,18-d_4-5-Hydroxy-prosta-6,14-dienoate (**31**)*

To a solution of lithium aluminum hydride (0.2 ml, 0.2 mmol, 1 M/THF) at 25°C was added a solution of absolute ethanol (0.997 ml, 0.2 mmol, 2 M/THF) dropwise over 10 min. Subsequently, (S)-binaphthol (57.3 mg, 0.2 mmol) in THF (0.25 ml) was added dropwise and the mixture was stirred at 25°C for 10 min. To the resulting (S)-BINAL-H solution was added the d_4-enone **30** (24 mg, 0.0399 mmol) in anhydrous THF (0.5 ml) at −98°C (liquid N_2/MeOH). The reaction mixture was stirred at −98°C for 2 h and then for another 2 h at −78°C. Methanol (0.5 ml) was added at −78°C to destroy the excess reducing agent and the mixture was allowed to warm to room temperature. After the addition of water (0.5 ml) and diethyl ether (3 ml), the reaction mixture was neutralized with 0.1 N HCl (0.3 ml). The resulting mixture was extracted with diethyl ether (2 × 10 ml). The organic phase was separated, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by flash chromatography using 10% ethyl acetate in hexane as an eluent to yield the

d_4-C_5-(S) hydroxy compound **31** (17.7 mg) as an oil: R_f = 0.25 (15% AcOEt in hexane). The enone **30** (2.5 mg) was also recovered; thus, the yield based on consumed starting material is 82%.

17,17,18,18-d_4(5S,6E,8β,9α,11α,14Z)-5,9,11-Trihydroxyprosta-6,14-dien-1-oic Acid and δ-Lactone (d_4-iPF$_{2\alpha}$-VI δ-Lactone) (32)

To a solution of the d_4-*bis*-silyloxy compound **31** (17.7 mg, 0.029 mmol) in anhydrous THF (1.5 ml) at 25°C was added a 1 M solution of tetrabutylammonium fluoride (0.294 ml, 0.29 mmol). The reaction mixture was stirred at 25°C for 8 h and quenched with saturated aqueous NH_4Cl (3 ml). The resulting mixture was extracted with ethyl acetate (2 × 10 ml). The organic layer was separated, washed with brine (5 ml), dried over Na_2SO_4, and concentrated *in vacuo* to give a mixture of d_4-iPF$_{2\alpha}$-VI **33** and the lactone **32** (10 mg, 91%) as an oil.

17,17,18,18-d_4(5S,6E,8β,9α,11α,14Z)-5,9,11-Trihydroxyprosta-6,14-dien-1-oic Acid (33)

To a solution of the mixture of d_4-iPF$_{2\alpha}$-VI **33** and the lactone **32** (10 mg, 0.027 mmol) in dioxane/water (1 ml/0.5 ml) at 0°C was added 5% aqueous KOH (1 ml). The reaction mixture was stirred at 0°C for 15 min and washed with ethyl acetate (2 × 0.5 ml) to remove any organic impurity present. The mixture was diluted with ethyl acetate (3 ml) and acidified with 5 wt% aqueous KH_2PO_4 (2 ml). The resulting mixture was extracted with ethyl acetate (2 × 15 ml). The extracts were washed with water (10 ml) and brine (10 ml) and dried over Na_2SO_4. Removal of the solvent afforded d_4-iPF$_{2\alpha}$-VI **33** (8.1 mg, 84%) as an oil: R_f = 0.325 (methanol/ethyl acetate/acetic acid 15/85/0.1).

^{1}H NMR (CD_3COCD_3). δ5.58–5.48 (m, 2H, C_7-H, C_6-H), 5.46 (m, 2H, C_{14}-H, C_{15}-H), 4.05 (m, 1H, C_5-H), 3.97 (m, 1H, C_9-H), 3.88 (m, 1H, C_{11}-H), 2.68 (m, 1H, C_8-H), 2.4 (q, J = 7.3 and 14.3 Hz, 1H, C_{10}-H), 2.3 (t, J = 7.3 Hz, C_2-H_2), 2.17–195 (m, 5H, C_{12}-H, C_3-H_2, C_{13}-H_2), 1.78–1.6 (m, 2H, C_{10}-H, C_4-H), 1.6–1.48 (m, 3H, C_4-H, C_{16}-H_2), 1.28 (bq, J = 7.2 and 14.4 Hz, 2H, C_{19}-H_2), 0.9 (t, J = 6.8 Hz, 3H, C_{20}-H).

^{13}C NMR (CD_3COCD_3). δ174.8, 136.7, 130.9, 129.8, 129.6, 76.2, 76.1, 72.4, 53.7, 51.5, 44.0, 37.9, 34.2, 32.3, 30.2, 29.3, 22.0, 14.4. ESI–MS *m/z* calc. for (M-1) 357, found 357.

(5S,6E,8β,9α,11α,14Z)-5,9,11-Trihydroxyprosta-6,14-dien-1-oic Acid δ-Lactone (iPF$_{2\alpha}$-I δ-Lactone) (32)

To a solution of iPF$_{2\alpha}$-VI **34** (4 mg, 0.0113 mmol) in methylene chloride/acetonitrile (0.7 ml/0.3 ml) at 0°C was added dicyclohexylcarbodiimide, DCC (23.3 mg, 0.113 mmol). The reaction mixture was stirred at room temperature for 3 h and quenched with saturated aqueous NH_4Cl solution (1 ml). The resulting mixture was extracted with methylene chloride (5 ml). The extract was dried with Na_2SO_4 and concentrated *in vacuo* to afford the title compound in quantitative yield. The residue was purified by flash chromatography using 3% methanol in methylene chloride as an eluent to give iPF$_{2\alpha}$-I δ-lactone **35** (2.5 mg, 66%) as an oil: R_f = 0.53 (15% methanol in methylene chloride).

^{1}H NMR ($CDCl_3$). δ5.68–5.58 (m, 2H, C_7-H and C_6-H), 5.5–5.42 (m, 1H, C_{14}-H), 5.41–5.32 (m, 1H, C_{15}-H), 4.8 (dt, J = 3.5 and 7.4 Hz, 1H, C_5-H), 4.05 (m, 1H, C_9-H), 4.0 (m, 1H, C_{11}-H), 2.84 (m, 1H, C_8-H), 2.6 (t, J = 6.8 and 13.7 Hz, 1H, C_2-H), 2.52 (t, J = 6.8 Hz, 1H, C_2-H′), 2.45 (m, 1H, C_{10}-H), 2.2 (m, 1H, C_{12}-H), 2.05–1.8 (m, 6H, C_3-H_2, C_{13}-H_2, C_{16}-H_2), 1.72–1.58 (m, 3H, C_4-H_2 and C_{10}-H′), 1.4–1.22 (m, 6H, C_{17}-H_2, C_{18}-H_2, C_{19}-H_2), 0.9 (t, J = 6.8 Hz, 3H, C_{20}-H_3).

^{13}C NMR ($CDCl_3$). δ171.1 (C1), 131.17 (C14), 130.75 (C6), 127.56 (C15), 79.83 (C5), 76.46 (C9 and C11), 53.7 (C8), 50.97 (C12), 42.4 (C2), 31.52, 29.51, 29.28, 28.46 (C4, C10, C13, C16), 27.42, 27.01, 22.55, 18.20 (C3, C17, C18, C19), 14.04 (C20). HRFAB–MS *m/z* calc. for $(M + Na)^+$ 359.2199, found 359.2192.

2. iPF$_{2\alpha}$-VI GC/MS Assays

Reagents

N,N-Diisopropylethylamine (DIPEA), pentafluorobenzyl (PFB) bromide, dodecane, *N,N′*-dicyclohexyl-carbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide methiodide (EDCM), and copper sulfate ($CuSO_4$) were purchased from Sigma Chemical Co. (St. Louis, MO). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Supelco, Inc. (Bellefonte, PA). Pyridine was purchased from Mallinckrodt Baker, Inc. (Paris, KY). All other solvents were obtained from Burdick & Jackson, Inc. (Muskegon, MI).

iPF$_{2\alpha}$-VI Assay Procedure (DCC Method)

To 1.5 ml urine was added 3.4 ng of d_4-iPF$_{2\alpha}$-VI **33** in 375 μl methanol (MeOH). Aqueous KOH solution (15%, 1.5 ml) was added and the sample was allowed to equilibrate at room temperature for 1 h. The pH was then adjusted to 2–3 with 1 N HCl, and the sample was centrifuged at 3000 rpm for 10 min. Two-thirds of the final solution was removed for solid-phase extraction (SPE) on a RapidTrace (Zymark Corp., Hopkinton, MA) using C18 EC (100 mg) cartridges (International Sorbent Technology, Mid Glamorgan, UK). The program used was as follows: the cartridge was conditioned with 3 ml EtOH at 2 ml/min and 0.5 ml 0.05 M phosphate buffer, pH 7, at 10 ml/min; the sample was loaded at 10 ml/min; the cartridge was rinsed with 1 ml pH 7 buffer,

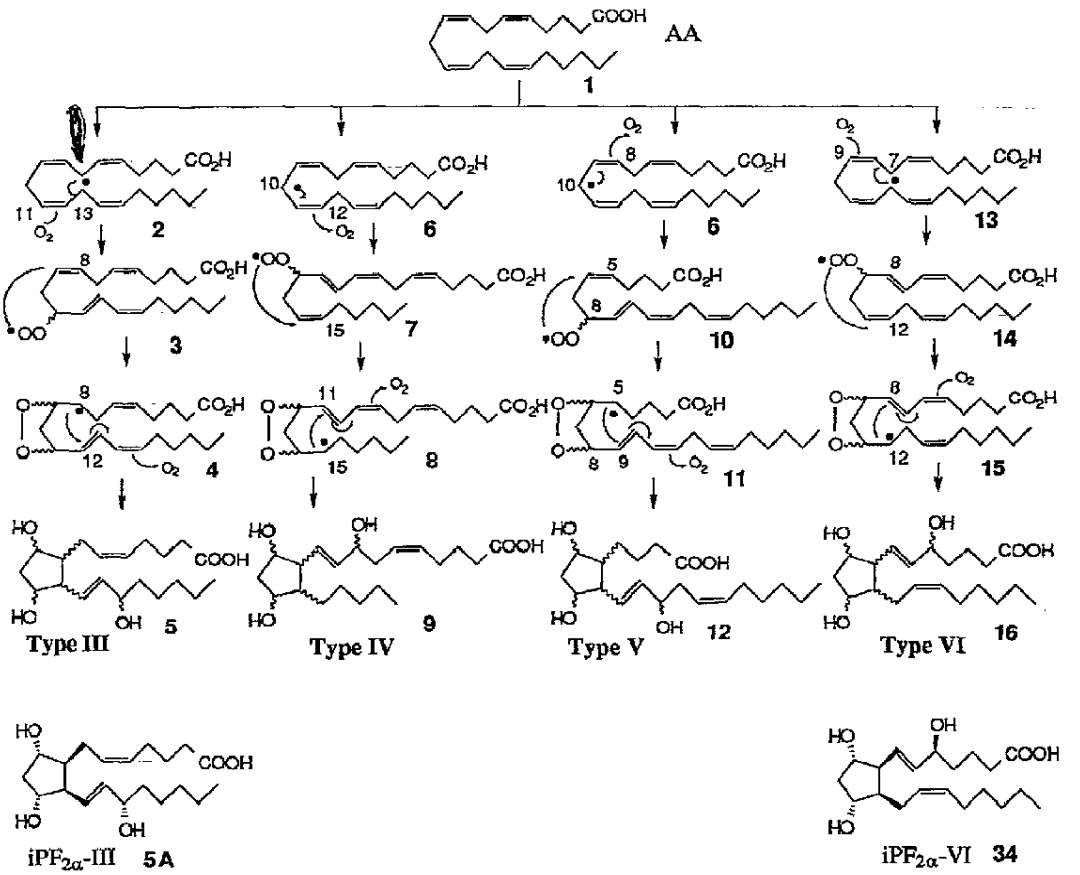


FIG. 1. Formation of four classes of isoprostanes from arachidonic acid.

10 ml/min, 1 ml 25% EtOH at 10 ml/min, air, 3 × 5 ml at 30 ml/min, and hexane, 3 × 5 ml at 15 ml/min; collect 1 ml EtOAc at 1 ml/min. The sample was then dried under a stream of N_2 and 35 μl DCC, 10 mg/ml in dichloromethane was added and allowed to stand for 30 min. The sample was spotted on a TLC plate (LK6D; Whatman, Inc., Clifton, NJ) and developed with a mobile phase of 3% MeOH in EtOAc. A separate plate on which had been spotted 2 μg iPF$_{2\alpha}$-VI **34** was simultaneously developed and visualized by dipping in 0.3 M $CuSO_4$ in 8% H_2PO_4 and heating on a hot plate. The appropriate zones 0.6 cm wide on the sample plates were scraped and the powder was extracted from 100 μl water with 1 ml EtOAc and dried under N_2. Ten microliters each of MeOH and 15% KOH was added and allowed to stand for 1 h, after which 100 μl 1 N HCl was added. The sample was extracted with 1 ml EtOAc and dried. Ten microliters of DIPEA and 20 μl 10% PFB were added, allowed to stand for 10 min, and dried. The sample was redissolved in 25 μl MeOH and applied to another TLC plate which was developed with EtOAc. A separate plate on which had been spotted 2 μg of iPF$_{2\alpha}$-IV PFB ester was developed and visualized as above. The sample zone, 0.6 cm, was scraped at the same R_f as the standard. Scrapings were extracted from 100 μl water with 1 ml EtOAc and dried. Ten microliters each of dry pyridine and BSTFA (Supelco, Bellefonte, PA) was added, allowed to stand for 10 min, and dried. The sample was then dissolved in 20 μl 1% BSTFA in dodecane for capillary gas chromatography/negative-ion electron-capture chemical ionization–mass spectrometric analysis. Each batch of samples included one water blank and one reagent blank.

iPF$_{2\alpha}$-VI Assay Procedure (EDCM Method)

The d_4-iPF$_{2\alpha}$-VI **33** (300 pg) was added as an internal standard to a urine sample (0.1 ml). EDCM (250 mg) was added and the sample allowed to stand at room temperature for 30 min. After SPE, the sample was purified by TLC and authentic iPF$_{2\alpha}$-VI lactone **35** was used as the visualization standard. The sample was saponified by 15% KOH/H_2O and neutralized by 1 N HCl. The sample was then derivatized as the PFB ester, purified by TLC, and converted to the TMS ether derivative. Finally the sample was analyzed by GC/MS.

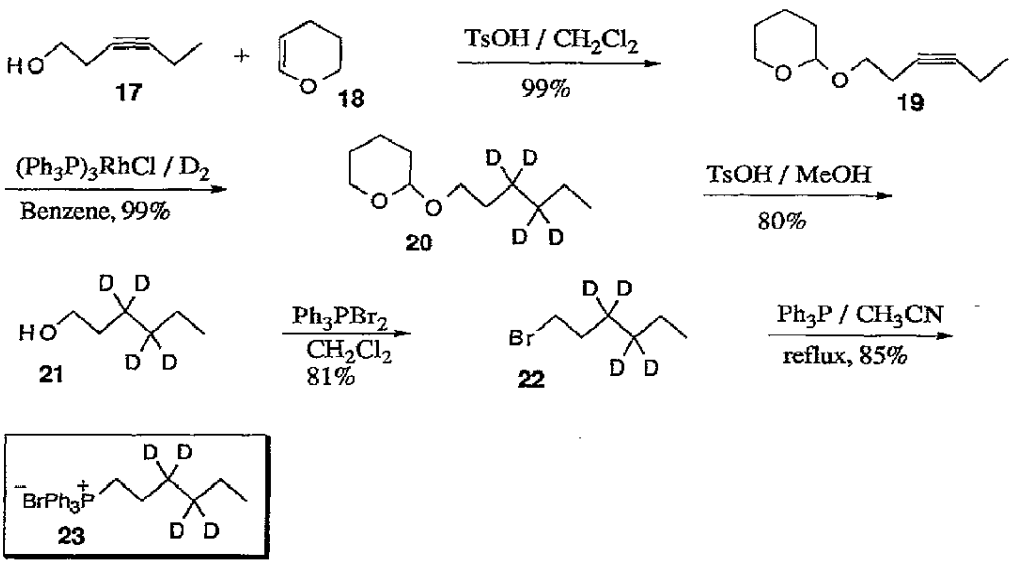


FIG. 2. Preparation of the d_4-hexyltriphenylphosphonium bromide from 3-hexynol.

Gas Chromatography/Mass Spectrometry

A Fisons MD-800 mass spectrometer equipped with a Fisons 8000 gas chromatograph and a Fisons AS-800 autosampler was used for all analyses. The MS was operated in the negative-ion electron-capture ionization mode, using ammonia as the moderating gas. Ions monitored were *m/z* 569 and 573 for $iPF_{2\alpha}$-VI and the internal standard, respectively. A 30-m, 0.25-mm-i.d., 0.25-μm-phase-thickness DB5-MS column was used with a temperature program of 1 min isothermal at 190°C, followed by heating at 20°C/min to 320°C. The carrier gas was helium.

RESULTS AND DISCUSSION

We have reported a new nomenclature of isoprostanes (24) which supersedes our older one. The main feature of the new nomenclature is the use of the ω-carbon of the polyunsaturated fatty acid as the basis for the classification of the different isoprostane groups. In the present paper $iPF_{2\alpha}$-VI and $iPF_{2\alpha}$-III are the new designations for the two isoprostanes we used to refer to as $IPF_{2\alpha}$-I and 8-*iso*-$PGF_{2\alpha}$ or $IPF_{2\alpha}$-IV, respectively. In the older nomenclature the four classes of iPs derived from arachidonic acid were classified as Types IV, III, II, and I. In the new nomenclature, we have assigned Groups III, IV, V, and VI, respectively, to the same four classes in order to correlate them with iPs derived from eicosapentaenoic acid and docosahexaenoic acid (DHA) (see Ref. 24 for a more detailed discussion).

Our original interest in $iPF_{2\alpha}$-VI **34** and its use as a potentially superior index of oxidant stress stemmed from the following considerations.

First, a look at the proposed four classes of iPs (Fig. 1) anticipated to be formed from AA by a free-radical oxygenation shows that only Group VI isoprostanes have an OH function on the C-5 relative to the COOH and are expected to easily form a six-membered-ring lactone (Fig. 1). That to us looked like a very positive feature and is in fact one of the reasons that we elected to undertake the total synthesis of $iPF_{2\alpha}$-VI early in the overall priority of the program. We thought that by performing a lactonization on an iP mixture from biological fluids, which we anticipated to be complex, Group VI would separate from the rest of the iPs, the lactones being much less polar than the unlactonized hydroxy acids of Groups III, IV, and V. This could lead to an improved and more efficient separation of Group VI iPs from the rest, which in turn could lead to a more sensitive and accurate assay for the measurement of iPs in biological fluids. This, in fact, turned out to be the case, as we are routinely using 0.1 ml of urine for $iPF_{2\alpha}$-VI measurement compared to 5 ml for the measurement of $iPF_{2\alpha}$-III.

Another factor which weighed heavily in our original decision to perform the synthesis of $iPF_{2\alpha}$-VI on a priority basis is also related to the positions of the OH group in relation to the carboxylic acid. We have in the past, while studying the metabolism of LTC_4, noticed that the majority of the β-oxidation occurred at the ω end of the molecule (25–28). This is counter to the accepted wisdom which gives preference to the carboxylic acid group already present in the molecule. A case in hand is the metabolism of prostaglandins in which the β-oxidation proceeds first with the COOH at C-1 before the ω end is affected. We interpreted this ob-

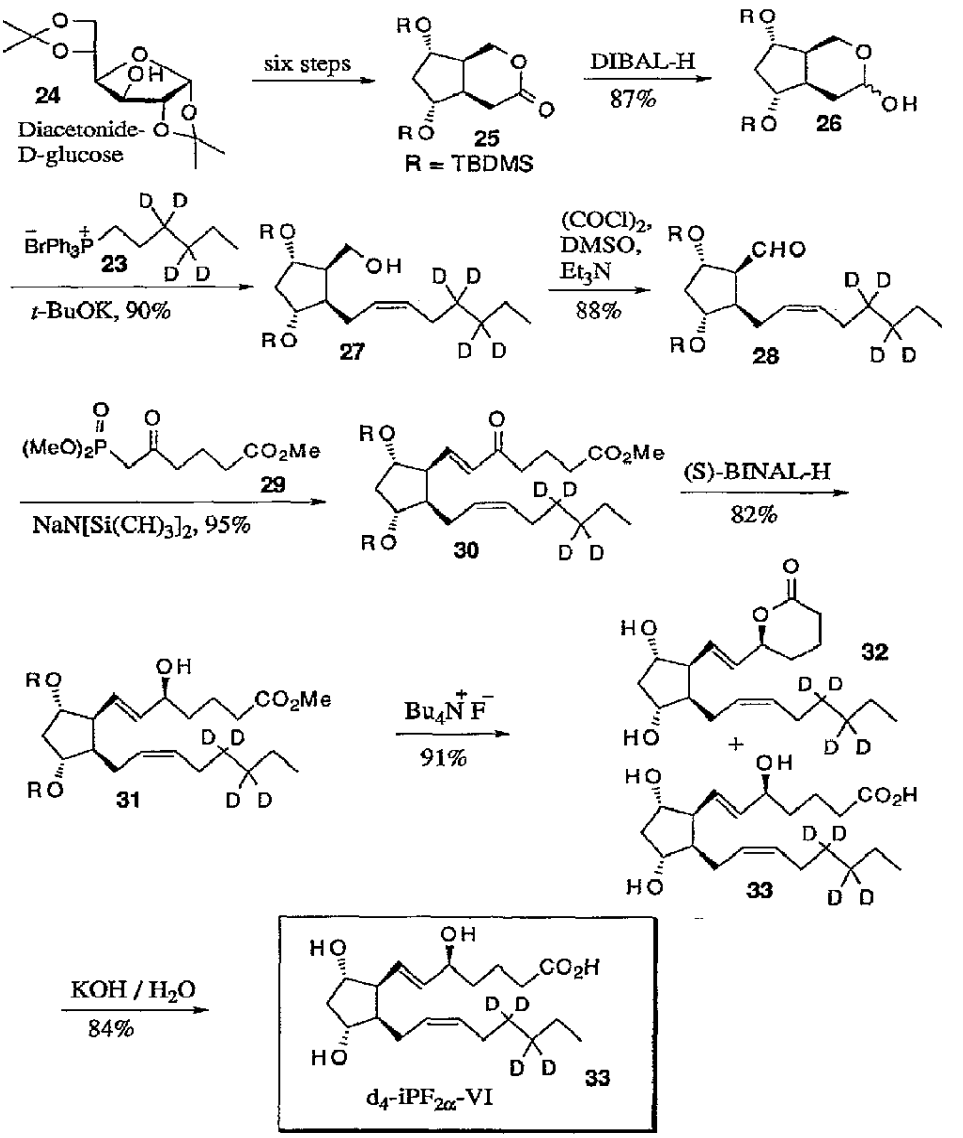
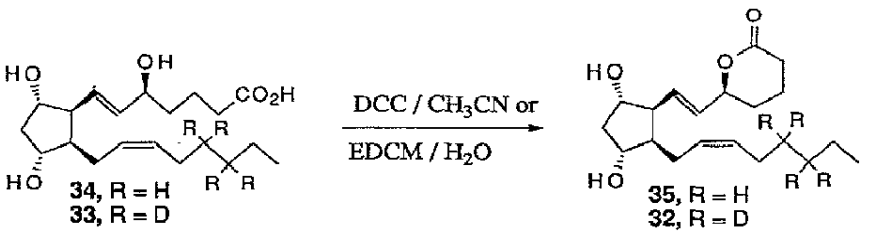


FIG. 3. Total synthesis of d_4-iPF$_{2\alpha}$-VI.

served preference in the case of LTC_4 as being due to the slowing of the metabolism at the C-1 end because of the presence of OH at the 5-position. Hence, we speculated that iPF$_{2\alpha}$-VI, if formed *in vivo,* would behave similarly and since β-oxidation at the ω end is a slower process, there would be a better chance of increased levels of iPF$_{2\alpha}$-VI in urine, making it easier to identify. As it turned out and as described later in this paper, iPF$_{2\alpha}$-VI is present in urine in substantial amounts. It is too early to tell if this speculation about the metabolism of iPF$_{2\alpha}$-VI, which originally helped us push forward our priorities, is in fact contributing to the high level of iPF$_{2\alpha}$-VI observed in urine.

Second, as was reported by us previously (23), iPF$_{2\alpha}$-VI appeared more abundant than iPF$_{2\alpha}$-III in human urine, an observation we have been able to confirm once the tetradeutero derivative was made by total synthesis, as described in the present paper. In fact, the level of iPF$_{2\alpha}$-VI in urine is several fold higher than iPF$_{2\alpha}$-III.

Third, the attraction of developing an isoprostane assay based on iPF$_{2\alpha}$-VI, or any isoprostane other than iPF$_{2\alpha}$-III for that matter, is that it bypasses problems attributable to COX-dependent formation of this particular F_2-iP, iPF$_{2\alpha}$-III. We and others have now demonstrated that both COX isoforms have the capacity to



A) Lactonization with DCC

1. Spike d_4-$iPF_{2\alpha}$-VI to 1.5 ml urine **2.** Acidify to pH 3 **3.** Solid Phase Extraction

4. Lactonize $iPF_{2\alpha}$-VI $\xrightarrow{DCC}$ $iPF_{2\alpha}$-VI δ-lactone **5.** Purify the lactones (TLC)

6. Saponify the lactones **7.** Extract $iPF_{2\alpha}$-VI with EtOAc **8.** Prepare PFB derivatives

9. Purify **10.** Prepare TMS derivatives **11.** Analyze by GC/MS

B) Lactonization with EDCM

1. Spike d_4-$iPF_{2\alpha}$-VI to 100 µl urine **2.** Lactonize $iPF_{2\alpha}$-VI $\xrightarrow{EDCM}$ $iPF_{2\alpha}$-VI δ-lactone

3. Purify the lactones by RP SPE and by TLC **4.** Saponify the lactones

5. Extract $iPF_{2\alpha}$-VI with EtOAc **6.** Prepare PFB derivatives **7.** Purify by TLC

8. Prepare TMS derivatives **9.** Analyze by GC/MS

FIG. 4. Assay procedures for $iPF_{2\alpha}$-VI using lactonization.

form $iPF_{2\alpha}$-III (9, 10, 29). This pathway is not only demonstrable *in vitro,* but also *ex vivo* as suppression of capacity for formation in serum in volunteers taking NSAIDs is observed. Actual *in vivo* generation, as reflected by urinary excretion of $iPF_{2\alpha}$-III, is unaffected (20). This implies that COX-dependent formation of $iPF_{2\alpha}$-III is likely to be a quantitatively trivial pathway of its formation *in vivo.* Nonetheless, COX activation is a common feature in human syndromes of oxidant stress, such as ischemia–reperfusion, cigarette smoking, and acute inflammation and targeting a different iP for analysis, e.g., $iPF_{2\alpha}$-VI, avoids any potential ambiguity or the necessity to resolve it by including NSAID control experiments. In addition, it has been recently shown that a substantial part of urinary $iPF_{2\alpha}$-III is COX dependent in rats but not in humans (30). For these reasons, an iP other than $iPF_{2\alpha}$-III would be a more logical target for an assay development.

We decided to introduce the deuterium label on the lower side chain. Figure 2 shows the design we used to introduce the four deuterium atoms. We used 3-hexynol **17** as the starting material for the preparation of (3,3,4,4-d_4)-hexylphosphonium bromide **23** (Fig. 2). 3-Hexynol **17** was converted to its tetrahydropyranyl ether **19** by treatment with 3,4-dihydro-2H-pyran **18**/*p*-toluenesulfonic acid (DHP/TsOH) in 99% yield. The resulting ether derivative **19** was deuterated catalytically with a tris(triphenylphosphine)chlororhodium complex [$(Ph_3P)_3RhCl$ Wilkinson's catalyst] (31, 32) to the tetradeutero THP ether **20** in 99% yield. The d_4-ether derivative **20** was transformed into (3,3,4,4-d_4)-hexyl bromide **22** by treatment with triphenylphosphonium dibromide (Ph_3PBr_2), after THP deprotection by TsOH/MeOH, in 65% overall yield. The reaction of the d_4-bromohexane **22** with triphenylphosphine in acetonitrile afforded (3,3,4,4-d_4)-hexyltriphenylphosphonium bromide **23** in 85% yield as shown in Fig. 2. An earlier attempt using Pd/C catalyst for the deuteration gave badly scrambled mixtures.

Figure 3 shows the completion of synthesis. Lactone **25** was prepared in six steps from commercial diacetonide D-glucose **24** as described by us previously (23). Di-isobutylaluminum hydride (DIBAL-H) reduction at −78°C afforded an 87% yield of the lactol **26.** The Wittig olefination was performed by using the *syn-anti-syn* bicyclic lactol **26** and (3,3,4,4-d_4)-hexyltriphenylphosphonium bromide **23** to give the d_4-olefinic compound **27** in 90% yield. The Swern oxidation of the d_4-alcohol **27** to aldehyde **28** was achieved in 88% yield using oxalyl chloride, DMSO, and triethylamine. The Horner–Emmons reaction of **28** with the phosphonate **29** to introduce the upper side chain was accomplished as follows. The anion of β-ketophosphonate **29** was generated with sodium *bis*(trimethylsilyl)amide in THF at room temperature and reacted at −78°C with aldehyde **28** to afford the enone **30** in 95% yield. The enantioselective reduction of the C-5 keto group in **30**

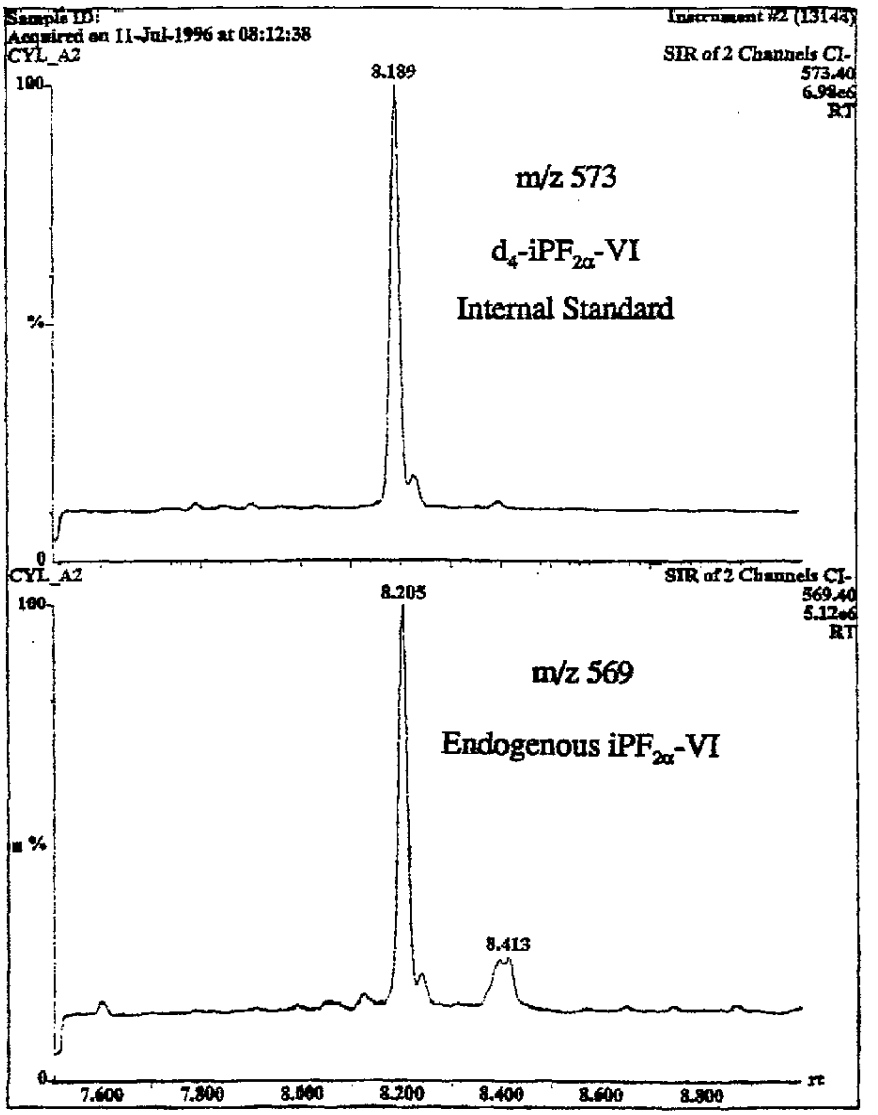


FIG. 5. Selected parallel ion monitoring of $iPF_{2\alpha}$-VI and its d_4-derivative in a normal human urine sample. The upper trace shows a peak at *m/z* 573 $(M - PFB)^+$ corresponding to authentic synthetic d_4-$iPF_{2\alpha}$-VI added as an internal standard. The lower trace shows endogenous $iPF_{2\alpha}$-VI in normal human urine (*m/z* 569) $(M - PFB)^+$ at the retention time (8.2 min).

with the chiral reducing agent (4, 33) (S)-BINAL-H proceeded well and afforded the desired pure 5(S) derivative **31** in 82% yield. The deprotection of the *bis*-silyl groups in **31** using tetrabutylammonium fluoride in THF at room temperature gave the lactone **32** and the acid **33.** Finally, the mixture of **32** and **33** without separation was treated with aqueous potassium hydroxide in dioxane at room temperature to yield the desired d_4-$iPF_{2\alpha}$-VI **33** in 97% yield.

As can be seen from Fig. 4, the lactonization of synthetic $iPF_{2\alpha}$-VI **34** is performed easily and in very high yield by procedures A and B. We have used procedures A and B in most of our measurements. The main difference between these two procedures is as follows. In procedure A, the lactonization step is performed after the purification of the urine sample. In procedure B, the lactonization with the water-soluble EDCM is done from the outset, directly in the urine sample. Procedure A is more economical as less cyclizing agent is used, making it cheaper to operate. Procedure B is shorter, but more expensive as a larger amount of EDCM is used because urine contains other chemicals which consume appreciable amounts of the cyclizing agent. By contrast, in procedure A, we use the cyclization step after the urine sample has been purified, resulting in a much lower consumption of DCC.

A typical selected ion-monitoring chromatogram for $iPF_{2\alpha}$-VI from human urine, spiked with the d_4-deriv-

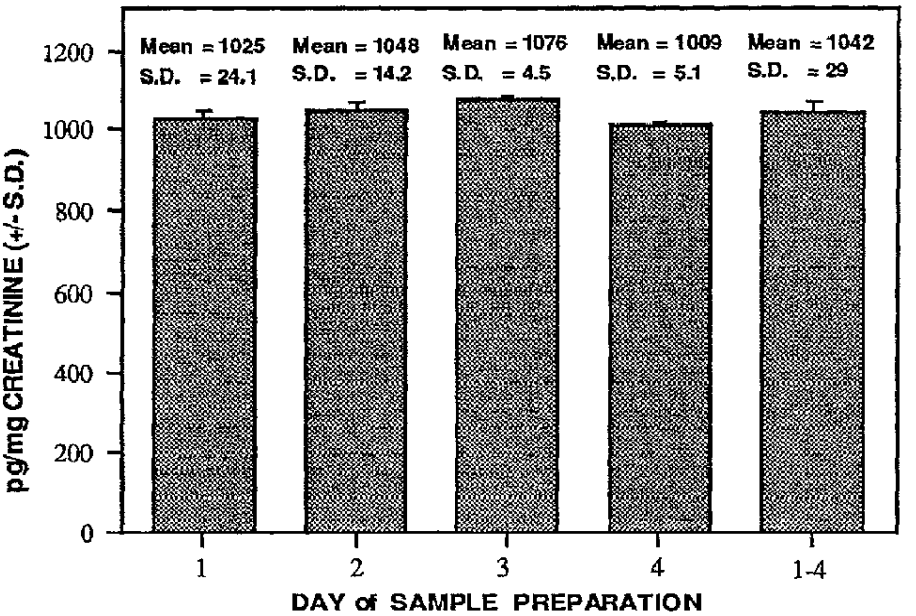


FIG. 6. Reproducibility of $iPF_{2\alpha}$-VI assay (DCC method). The same urine sample was assayed six times on each of 4 days. The bar for days 1–4 shows the overall mean for all the samples analyzed.

ative and isolated by any of the two procedures, is shown in Fig. 5. The d_4 compound shows a slightly shorter retention time, which is often observed for polydeuterated derivatives.

The reproducibility of the assay by the two procedures A and B is excellent. In procedure A, the same urine sample was worked up six times on each of 4 days. The standard deviation on each of these days represents the intraassay variability (Fig. 6). Totaling all the samples ($n = 24$) gives the interassay variability (Fig. 6). In procedure B, Fig. 7, six determinations a day were performed on 3 successive days. Column 4

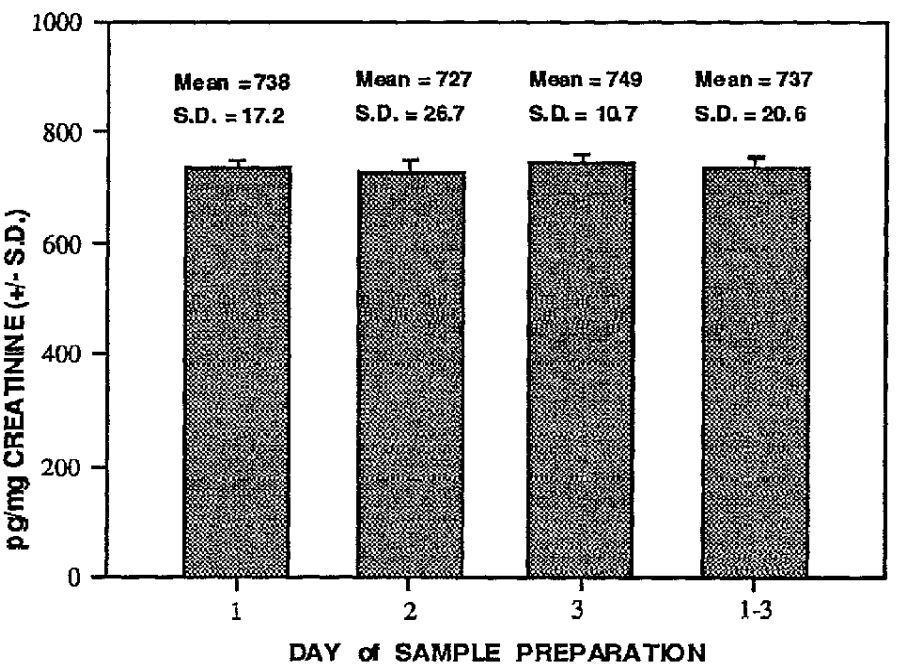


FIG. 7. Reproducibility of $iPF_{2\alpha}$-VI assay (EDCM method). The same urine sample was assayed six times on each of 3 days. The bar for days 1–3 shows the overall mean for all the samples analyzed.

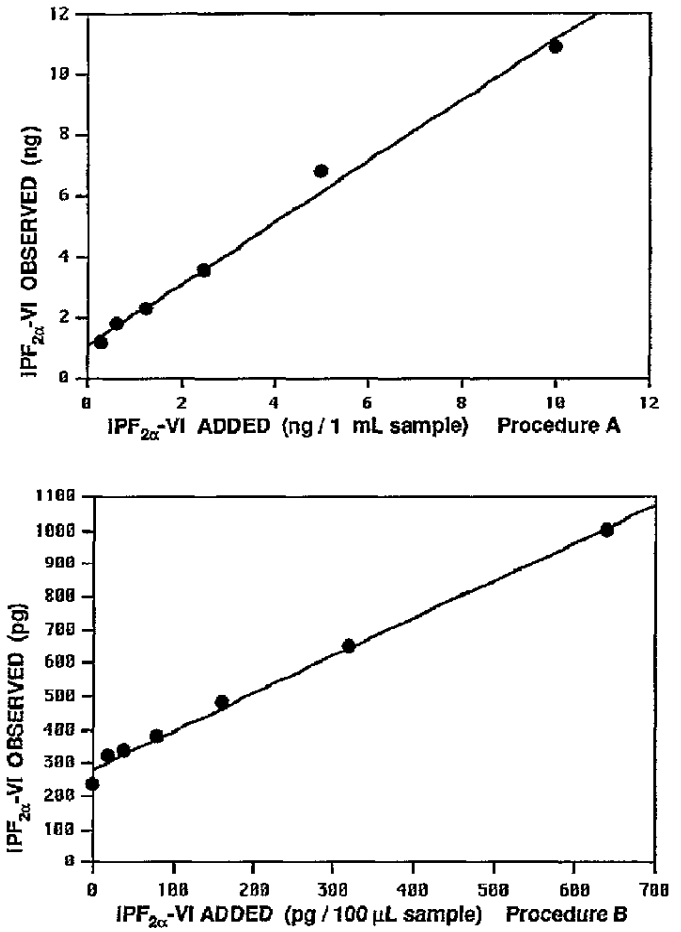


FIG. 8. (Top) Linearity of iPF$_{2\alpha}$-VI assay by DCC method. Increasing amounts (0.31–10 ng) of iPF$_{2\alpha}$-VI added were plotted against the observed iPF$_{2\alpha}$-VI. (Bottom) Linearity of iPF$_{2\alpha}$-VI assay by EDCM method. Increasing amounts (20–640 pg) of iPF$_{2\alpha}$-VI added were plotted against the observed iPF$_{2\alpha}$-VI.

shows the interassay variability and is the average of the 18 measurements from columns 1–3.

Figure 8, top, shows the linearity of the assay using procedure A. Various amounts of synthetic iPF$_{2\alpha}$-VI were added to the urine, starting with 0.31 ng and doubling the dose each time. Figure 8, bottom, shows the linearity of the assay by the EDCM procedure. Synthetic sample addition starts at 20 pg and doubles for each successive point.

As mentioned earlier, this type of isoprostane is unique in that it possesses a hydroxyl group on C-5 which enables it to form a cyclic six-membered-ring lactone structure. The lactonization process, which we took advantage of, has allowed us to separate the iPF$_{2\alpha}$-VI lactone from the other isoprostanes and resulted in improvement in the GC/MS procedure. In the past we have used the TBDMS derivatives for the identification of iPF$_{2\alpha}$-III and iPF$_{2\alpha}$-VI. The reason was that the TBDMS derivatives provided a better separation of the isoprostanes, but at a price: the sensitivity of the assay is reduced. By using the lactonization procedure, we have been able to switch to a TMS derivative for iPF$_{2\alpha}$-VI which offers faster reaction times, shorter GC retention times, and higher sensitivity, which, in turn, enables us to use, if necessary, sample volumes as low as 100 µl.

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